Development and Validation of a Novel 3'3'-cGAMP Monoclonal ELISA

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KEY FINDING

A highly sensitive and specific ELISA to 3'3'-cGAMP that correlates well with LC-MS/MS.

INTRODUCTION

Second messengers are small molecules that cause a wide variety of cellular changes transcriptionally, translationally, and post-translationally. 3'3'-cGAMP is a bacterial second messenger produced from ATP and GTP by specific dinucleotide cyclases.[1] It is produced and regulated *via* two different pathways, which use distinct classes of synthases, effectors, and phosphodiesterases (PDEs).[2,3] 3'3'-cGAMP binds to riboswitches to regulate motility, biofilm formation, virulence, and colonization through gene transcription.[4] The study of cyclic dinucleotides (CDNs) in bacterial innate immunity is a growing area of research. Current detection methods utilize mass spectrometry, which can be both costly and timely. In addition to that, the isolation and purification of CDNs can be complicated and tedious. Rapid and accurate detection methods are critical to enable researchers to study and/or identify the relevant biological pathways.

This poster will focus on the development and validation of a novel 3'3'-cGAMP monoclonal enzyme-linked immunosorbent assay (ELISA). The data provided will demonstrate a highly sensitive and specific ELISA validated by LC-MS/MS.

METHODS

Immunogen Conjugation

Three isomers of 3'3'-cGAMP were conjugated to thyroglobulin and each injected into three mice and three rabbits each, giving a total of 18 animals. Bleeds from those 18 animals were functionally screened by the ability to bind three different isomers of 3'3'-cGAMP-horseradish peroxidase (HRP). Positive antibody titers were obtained from all animals, indicating a specific antibody response to 3'3'-cGAMP.

Monoclonal Antibody

All mice received an initial injection followed by two monthly boosters. Antibody titers were checked ten days after each booster and functionally screened with all three HRP tracers. Two of the three immunogens resulted in one mouse chosen for hybridoma production. Greater than 100 parental lines were selected for further characterization by screening supernatants from the hybridoma cell lines with two HRP tracers. Further characterization of the parental lines included sensitivity and specificity testing of the antibody response. Only two parental lines met internal specifications. These parental lines were selected to move forward, sub-cloned by limiting dilution, and purified for assay development. The antibody for this ELISA was finally chosen based on performance characteristics after extensive testing of both monoclonal antibodies with native samples.

Principle of the Assay

This ELISA is based on competition between free 3'3'-cGAMP and a 3'3'-cGAMP-HRP conjugate (tracer) for a limited number of 3'3'-cGAMP monoclonal antibody binding sites. Because the concentration of the HRP tracer is fixed in the well, the concentration of free 3'3'-cGAMP, either found in standard or sample wells, will be inversely proportional to the absorbance.

This is a two-step ELISA. In the first step, the standards, samples, tracer, and antibody are added to the wells and incubated for two hours. Following the incubation, the plate is washed to remove any unbound reagents and TMB substrate is added. The plate is allowed to develop before stop solution is added and absorbance is read at 450 nm.

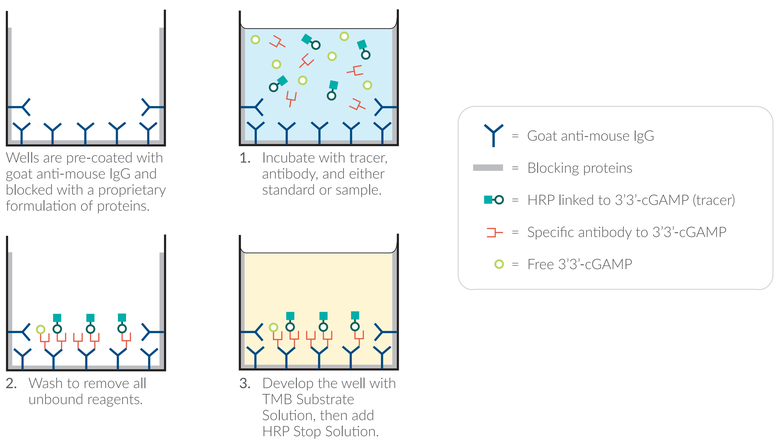


FIGURE 1 – Schematic of the 3'3'-cGAMP ELISA Kit

METHODS CONTINUED

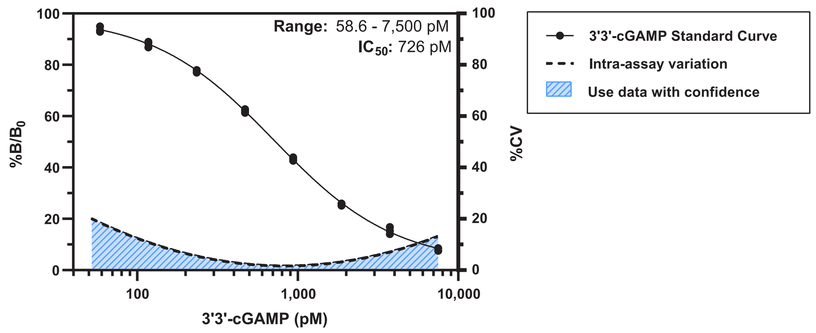
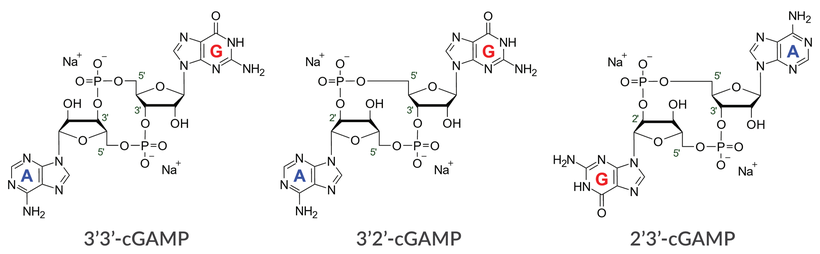


FIGURE 2 – Optimized standard curve for the 3'3'-cGAMP ELISA Kit

ELISA VALIDATION

Specificity



Compound	Cross Reactivity (%)
3'3'-cGAMP	100
3'2'-cGAMP	0.018
2'3'-cGAMP	0.006
Cyclic di-GMP	0.004
2'2'-cGAMP	0.002
pApG	0.002

Compound	Cross Reactivity (%)
ATP	<0.001
GTP	<0.001
AMP	<0.001
GMP	<0.001
cAMP	<0.001
cGMP	<0.001

FIGURE 3 – Specificity of the 3'3'-cGAMP ELISA
Structurally similar and related compounds were tested for cross reactivity in the ELISA. These compounds were serially diluted in assay buffer, evaluated in the ELISA, and compared to the standard curve using the formula below.

$$\%\ Cross\ Reactivity = \left[\frac{50\%\ B/B_0\ value\ for\ the\ 3'3' - cGAMP\ Standard\ Curve}{50\%\ B/B_0\ value\ for\ the\ potential\ cross\ reactant}\right] * 100\%$$

Parallelism & Linearity

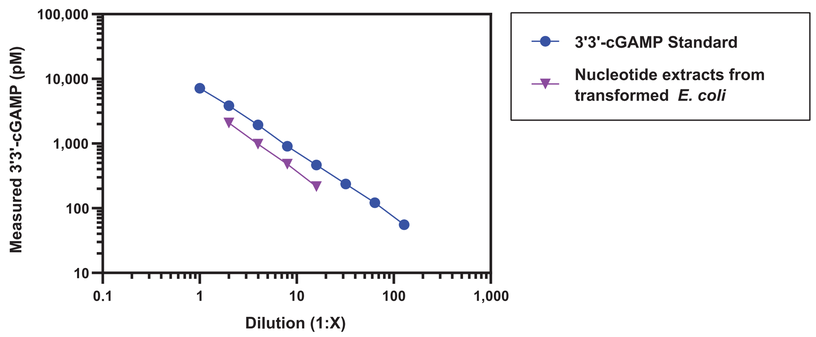


FIGURE 4 – The 3'3'-cGAMP ELISA Kit shows parallelism between samples and the standard curve
E. coli was transformed with DncV, stimulated for 3'3'-cGAMP production, and purified. Nucleotide extracts were diluted with Immunoassay Buffer C and evaluated in the 3'3'-cGAMP ELISA Kit. Concentrations of measured 3'3'-cGAMP were plotted as a function of dilution factor and compared to the standard.

ELISA VALIDATION CONTINUED

Dilution Factor	Measured Concentration (nM)	Linearity (%)
E. coli Lysates		
800	1,726	100
1,600	1,748	101
3,200	1,795	104
6,400	1,718	99.5
PC3 Lysates		
800	1,570	100
1,600	1,513	96.3
3,200	1,522	96.9
6,400	1,465	93.2
Jurkat Supernatant		
800	1,865	100
1,600	1,707	91.5
3,200	2,060	110
6,400	1,873	100

FIGURE 5 – The 3'3'-cGAMP ELISA Kit shows excellent dilutional linearity
E. coli lysates in B-PER™ (Thermo Scientific), PC3 lysates in M-PER™ (Thermo Scientific), and Jurkat cell supernatant were spiked with 1,600 nM 3'3'-cGAMP, serially diluted with Immunoassay Buffer C, and evaluated for linearity in the 3'3'-cGAMP ELISA Kit.

Spike & Recovery

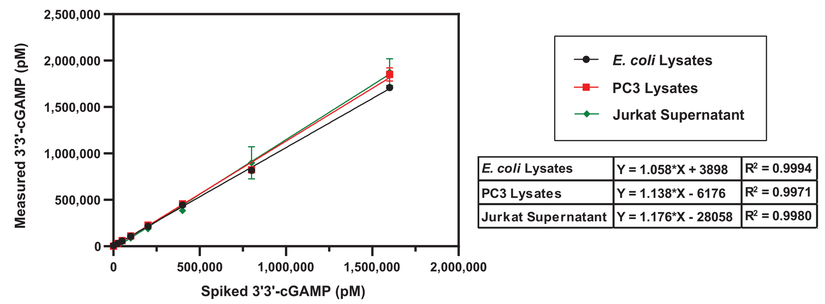


FIGURE 6 – Spike and recovery in various matrices
E. coli lysates in B-PER™ (Thermo Scientific), PC3 lysates in M-PER™ (Thermo Scientific), and Jurkat cell supernatant were spiked with different amounts of 3'3'-cGAMP, serially diluted with Immunoassay Buffer C, and evaluated using the 3'3'-cGAMP ELISA Kit. The error bars represent standard deviations from multiple dilutions of each sample.

LC-MS/MS Correlation

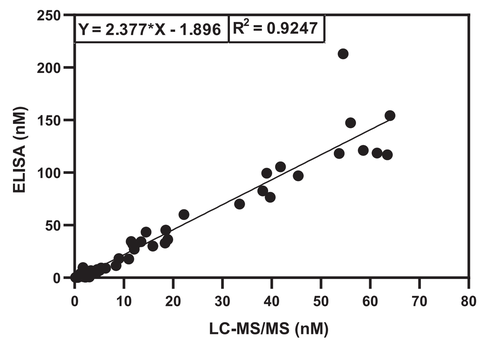


FIGURE 7 – Excellent correlation of the 3'3'-cGAMP ELISA to LC-MS/MS
Transformed *E. coli* samples were generated and measured by LC-MS/MS in the Department of Microbiology & Molecular Genetics by the Waters Lab at Michigan State University and compared to values measured by Cayman's 3'3'-cGAMP ELISA Kit.[5] ELISA values were obtained from multiple dilutions of each sample.

CONCLUSIONS

Cayman's novel 3'3'-cGAMP ELISA Kit (Item No. 502130) provides an easy-to-use tool for measurement of 3'3'-cGAMP in bacterial and mammalian cell lysates and cell supernatants. Validation of this ELISA has shown outstanding correlation to measurement by LC-MS/MS and allows researchers an inexpensive method to study and identify relevant biological pathways.

Download this poster & learn more about our novel 3'3'-cGAMP Monoclonal ELISA

Acknowledgements

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